

Dual Fluorochrome Flow Cytometric Assessment of Yeast Viability

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Abstract A novel staining protocol is reported for the assessment of viability in yeast, specifically the biocontrol yeast, *Pichia anomala*. Employing both the red fluorescent membrane potential sensitive oxonol stain DiBAC₄(5) (Bis-(1,3-dibutylbarbituric acid)pentamethine oxonol), a structural analog of the commonly used DiBAC₄(3) (Bis-(1,3-dibutylbarbituric acid)trimethine oxonol), with one of the esterase dependent green fluorogenic probes such as CFDA-AM (5-Carboxyfluorescein diacetate, acetoxy-methyl ester) or Calcein-AM (Calcein acetoxyethyl ester), a two-color flow cytometric method was developed, which yields rapid quantitative information on the vitality and vigor of yeast cell cultures. The method was validated by cell sorting and analysis of live, heat killed, and UV-treated yeast.

Introduction

Flow cytometric methods for assessing cell viability frequently employ one or more fluorescent probes for parameters such as membrane integrity, enzymatic activity, nucleic acid content, and membrane potential. In general, it is desirable when two or more probes are used

simultaneously, that they not duplicate positive or negative indication for the same parameter. It is desirable to combine a probe that is positive for a vital parameter (e.g., enzymatic activity) with one that is negative for another (e.g., membrane integrity). In this way viable or vital cells are clearly discerned by development of fluorescence from one probe versus the other. Greater confidence in interpretation of results is achieved by use of more than one probe [3].

Numerous fluorescent probes of membrane potential have been employed in flow cytometry, including the cyanine dyes, rhodamine, and the oxonol stains [16, 18]. A favorable compound used to estimate cell viability of various organisms and particularly yeasts is the oxonol DiBAC₄(3) [1, 4, 11]. It has been employed, for e.g., to monitor yeast vitality during cider fermentation [5], to study heavy metal toxicity in *Candida* sp. [20], as well as to study oxidative and nitrosative stress in protists [12], and as a rapid test for methicillin-resistant *Staphylococcus aureus* [19].

Oxonol stains are lipophilic anionic compounds which are capable, unlike most ionic molecules, of entering the plasma membrane barrier. DiBAC₄(3) fluorescence is strongly dependent upon its environment [2], increasing with hydrophobicity [6]. The plasma membrane in vital cells will normally exhibit a potential gradient such that the exterior is more positively charged and the oxonol will accumulate toward the outer surface [13]. Under this condition, the fluorescence is suppressed. However, when membrane potential collapses, whether in response to some cytotoxic event or otherwise, fluorescence increases. This response has been modeled according to Nernstian proportioning of the stain in response to membrane potential [7, 17].

The biocontrol yeast *Pichia anomala* WRL-076 [9, 10] is applied as a foliar spray to pistachio and almond trees for

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control of *Aspergillus flavus*. Therefore, it is naturally exposed to environmental stress such as temperature fluctuation, desiccation, water availability, and UV irradiation. An accurate and rapid method to monitor the viability of yeast under such environmental stresses is needed to facilitate the formulation of more effective and durable biocontrol yeast products.

We report the application of the red fluorescent oxonol stain, DiBAC₄(5), in conjunction with green fluorescent esterase indicating probes such as Calcein-AM and CFDA-AM to study the viability of the biocontrol yeast *P. anomala*.

Methods

Strains and Culture

Pichia anomala strain WRL-076, a saprophytic yeast isolate from nut trees [9], was maintained on DIFCO™ Potato Dextrose Agar (PDA) and cultured in DIFCO™ Potato Dextrose Broth (PDB) at 28°C. Long-term liquid storage was maintained in isotonic glycerol solution (0.9% w/v NaCl and 5% v/v glycerol) at 4°C.

Temperature and UV Irradiation Treatment

Yeast cells grown overnight in NYDB (8 g/l DIFCO™ Nutrient Broth, 5 g/l DIFCO™ Yeast Extract, 10 g/l Dextrose) at 28°C were used as live cell controls. A portion was incubated at 80°C for 5 min to generate dead cells as controls. Spiral plating using an Autoplate 4000 system (Spiral Biotech, Norwood, MA) verified that survival of heat-treated samples was nil after at least 3 min at 80°C. Yeast suspensions were uniformly exposed to UV irradiation by placement in open petri dishes located symmetrically under two 15 W germicidal UV lamps (Sankyo Denki G15T8, 4.9 W 253.7 nm UV output per lamp) at a distance of 5 cm. Estimated UV fluence rate was about 5 mW/cm². Samples were collected at 5, 10, 15, and 30 min for flow cytometric analysis and spiral plating.

Flow Cytometry

Flow cytometric equipment comprised a FACSVantage SE flow cytometer and cell sorter (BD Biosciences, San Jose, CA) with Enterprise II argon laser (water cooled, Coherent, Santa Clara, CA) with 488 nm emission. Filter set included 530/30 nm bandpass filter for “green” fluorescence in channel FL1 and 630/22 nm bandpass filter for “red” fluorescence in channel FL3. Data collection and analysis were performed using BD CellQuest software, and cell sorting was performed directly to petri plates using an

auxiliary fixture controlled by BD CloneCyte software. Cell sorting was of single cell events onto PDA petri plates in a 10 by 10 grid pattern (100 cells per plate). Data on 10,000 individual cells were collected per sample analyzed.

Fluorescent Stains

Stock solutions of the following were prepared in dimethyl sulfoxide (DMSO): 2.5 mg/ml (4.7 mM) CFDA-AM (5-Carboxyfluorescein diacetate, acetoxyethyl ester), 1 mg/ml (1.9 mM) DiBAC₄(3) (Bis-(1,3-dibutylbarbituric acid) trimethine oxonol), 1 mg/ml (1.8 mM) DiBAC₄(5) (Bis-(1,3-dibutylbarbituric acid)pentamethine oxonol), 1 mg/ml (2.3 mM) DiSBAC₂(3) (Bis-(1,3-diethylthiobarbituric acid) trimethine oxonol), 1 mg/ml (1.0 mM) Calcein-AM (Calcein acetoxyethyl ester), 1 mg/ml (3.1 mM) Nile Red, 20 mM PI (Propidium Iodide), 3 mM DiOC₂(3) (3,3'-diethyloxacarbocyanine iodide). All stains were obtained from Anaspec (Fremont, CA).

All samples were stained in PBS (phosphate buffered saline) unless otherwise indicated. Alternative staining media tested included NYDB, Tris-Mg²⁺ buffer (1.21 g/l Tris base, 1 g/l MgCl₂ and adjusted to pH 7 with 1 M HCl) [11] and water. Yeast samples were diluted with staining medium to between 10⁶ and 10⁷ cells per ml prior to staining. Staining was performed by addition of 1 µL of the requisite stain(s) per 1 ml of sample and held for 30 min at room temperature in the dark. Samples were thereafter kept on ice until analyzed or sorted.

Results

Test of Suitability of Commonly Used Vital Stains

Our aim was to identify a staining protocol for our chosen yeast to best estimate viability and perhaps the causes of lost viability. Initially, we surveyed a variety of stains for their differential fluorescence uptake by live and heat-killed *P. anomala* and found the most marked shift in fluorescence using DiBAC₄(3) or propidium iodide (see Table 1). The esterase dependent probes CFDA-AM and Calcein-AM did not initially appear very promising because of relatively poor uptake by live yeast. Nile Red, was strongly fluorescent in dead cells with an apparent spectral shift toward the green channel.

Of the stains tested, DiBAC₄(3) and PI were both fluorescent in non-viable cells. With these two compounds, tests were performed in several staining media; PBS, NYDB, Tris-Mg²⁺, and water. Figure 1 shows the results on the fluorescence of live and heat-killed yeast stained with these stains in the different media. With the exception of water as a staining medium, there was little effect on the

Table 1 Staining intensity of live and dead *P. anomala* with various stains

Stain	Relative fluorescence intensity			
	Live yeast		Dead yeast	
	FL1	FL3	FL1	FL3
DiOC ₂ (3)	+	+	+	+
DiBAC ₄ (3)	-	-	++	++
DiBAC ₄ (5)	-	-	++	++
PI	-	-	++	++
CFDA-AM	+	+	-	-
Calcein-AM	+	+	-	-
Nile red	-	+	++	++

FL1 “green” channel, FL3 “red” channel

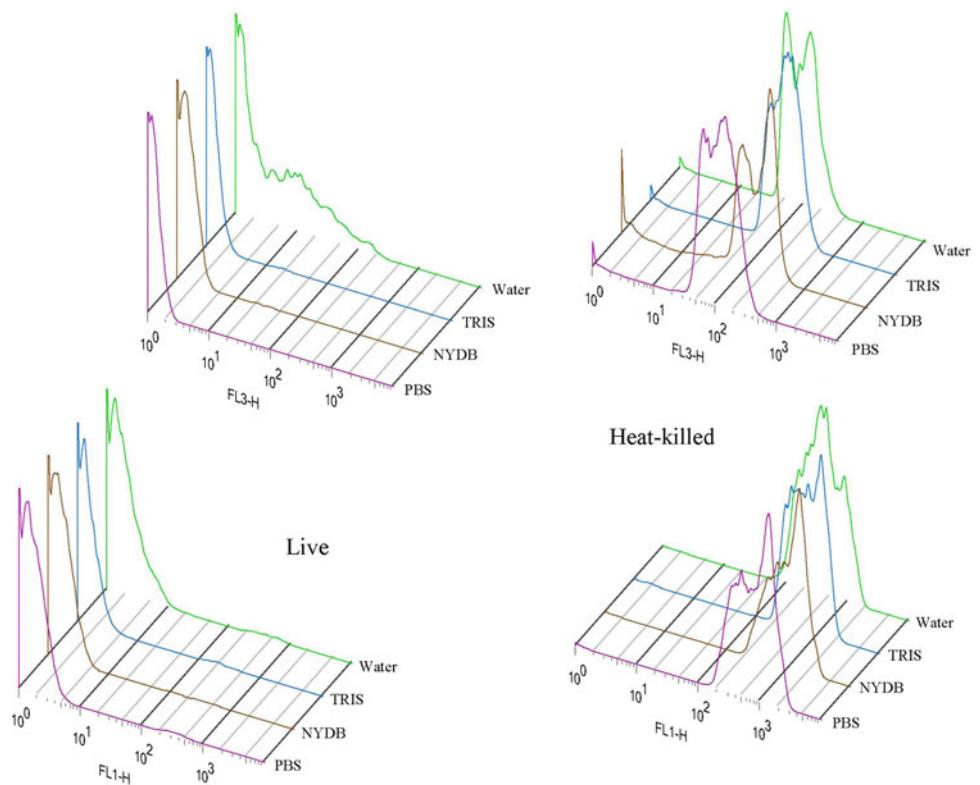
- no or minimal fluorescence

+ low fluorescence

++ strong fluorescence

quality of staining. In water, a population of the “live” cells took up some PI. This is presumably the result of osmotic stress. However, there was not a corresponding significant shift in DiBAC₄(3) fluorescence.

The two esterase-dependent fluorogenic stains, CFDA-AM and Calcein-AM, exhibit positive correlation to viability but the fluorescence shift is not as dramatic as for DiBAC₄(3) and PI. However, all of these compounds fluoresce predominantly green with the exception of PI.

Fig. 1 Comparison of effect of staining medium on uptake of PI (top figures) and DiBAC₄(3) (bottom figures) by live (left figures) or heat-killed (right figures) *P. anomala*

Evaluation of DiBAC₄(5)

In order to develop a dual stain protocol utilizing one of the membrane potential stains in conjunction with one of the esterase dependent stains, an alternative compound was needed which did not fluoresce predominantly green. Those considered included DiSBAC₂(3) and DiBAC₄(5), which is a structurally related analog of DiBAC₄(3). We found that DiSBAC₂(3) labeled cells exhibited a relatively large signal in the green channel making it unsuitable in conjunction with either CFDA-AM or Calcein-AM, which stain relatively dimly under normal conditions. With this combination careful signal compensation would be required. The focus then was placed on DiBAC₄(5), which produced little signal in the green channel, making compensation more simple.

Figure 2 shows an example plot of fluorescence in the red channel versus the green channel for a sample of live cells stained with DiBAC₄(5) and Calcein-AM. Noteworthy in this plot is that bulk of the cells, presumably the viable cells, are situated in the far lower left portion of the plot, where there is very little red signal and relatively small green signal. A second population is discernable mainly above the first, primarily exhibiting red fluorescence. There are scattered other cells with relatively high signal in both channels. Cells were sorted from regions in the different quadrants and confirm that 96% of those sorted from the lower left were viable, 65% of those from

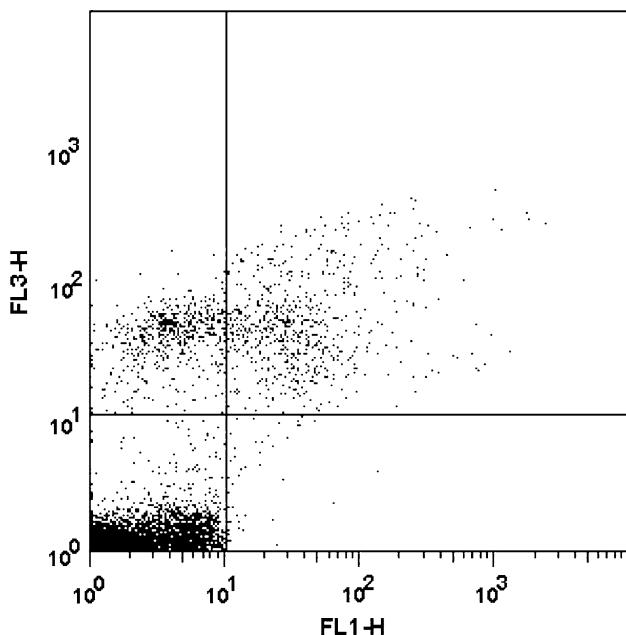


Fig. 2 Plot of *P. anamola* stained with 1 $\mu\text{g/ml}$ (1.8 μM) DiBAC₄(5) (“red” channel FL3) and 1 $\mu\text{g/ml}$ (1.0 μM) Calcein-AM (“green” channel FL1) in PBS

the lower right were viable and less than 3% from elsewhere on the plot. This indicates there is a strong correlation between the development of red fluorescence, i.e., loss of cell membrane potential, and the loss of cell viability. On the other hand, development of green fluorescence, access of Calcein-AM to esterases, is not a reliable indicator, by itself, of cell vitality. It is hypothesized that green signal may increase in cells which have weakened cell membranes, allowing more of the substrate into the cells.

A comparable experiment employing DiBAC₄(5) in conjunction with CFDA-AM is illustrated in Fig. 3. In this case, it is seen that the general level of green signal is higher with this particular compound (CFDA-AM), and again, the bulk of the cells have very little red fluorescence. Again, there are cells exhibiting rather strong signals in both channels (seemingly enzymatically active, yet lacking an ability to maintain a plasma membrane potential). Cells were sorted from the respective quadrants, as before. In this case, based on duplicate plates, 17% of those in the lower left quadrant formed colonies, 82% in the lower right quadrant formed colonies, 6% in the upper right quadrant and 0% in the upper left quadrant. This shows that development of red fluorescence is strongly correlated with loss of viability. Figure 4 illustrates the development of fluorescence of these two stains in live and heat-killed controls compared with unstained yeast.

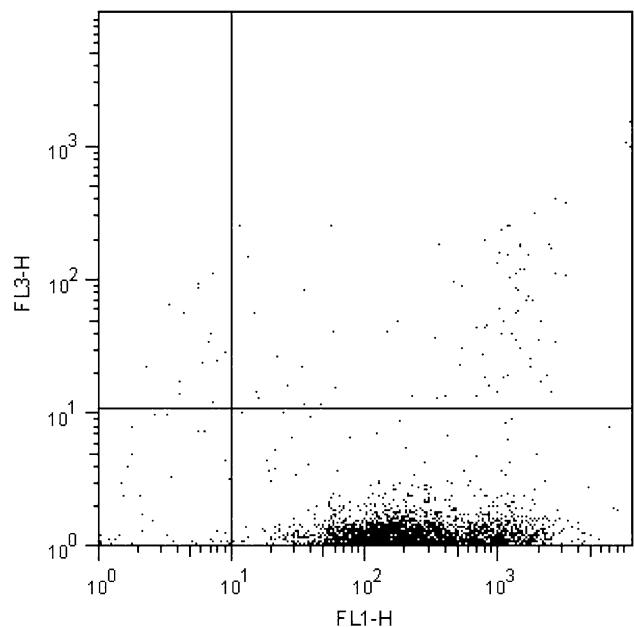


Fig. 3 Plot of *P. anamola* stained with 1 $\mu\text{g/ml}$ (1.8 μM) DiBAC₄(5) (“red” channel FL3) and 2.5 $\mu\text{g/ml}$ (4.7 μM) CFDA-AM (“green” channel FL1) in PBS

Effect of UV Irradiation on Yeast Cells

Utilizing the DiBAC₄(5)/CFDA-AM protocol, an experiment was performed in which samples of yeast in PBS were exposed to radiation from two germicidal UV lamps. The yeast suspensions were placed in open petri dishes symmetrically under the lamps for uniform exposure. Figure 5 illustrates the time course of this experiment. Initially, most cells fluoresce moderately green with a few exhibiting some red fluorescence. Immediately after commencement of UV exposure a population develops which exhibits much red fluorescence reflecting collapsed membrane potential. At the same time, there is a shift in the initial population to higher green fluorescent values and some cells exceed even those fluorescence levels while also exhibiting high red fluorescence. As time progresses, the majority of cells lose green fluorescence but maintain red fluorescence. Figure 6 illustrates the loss of culturability of the yeast with UV exposure. By 30 min almost no colonies are formed. This corresponds with the loss of the population expressing low DiBAC₄(5) fluorescence.

Discussion

We found the combination of the stains DiBAC₄(5) and CFDA-AM to be particularly amenable to this yeast. DiBAC₄(5) has little spill over into the green fluorescence

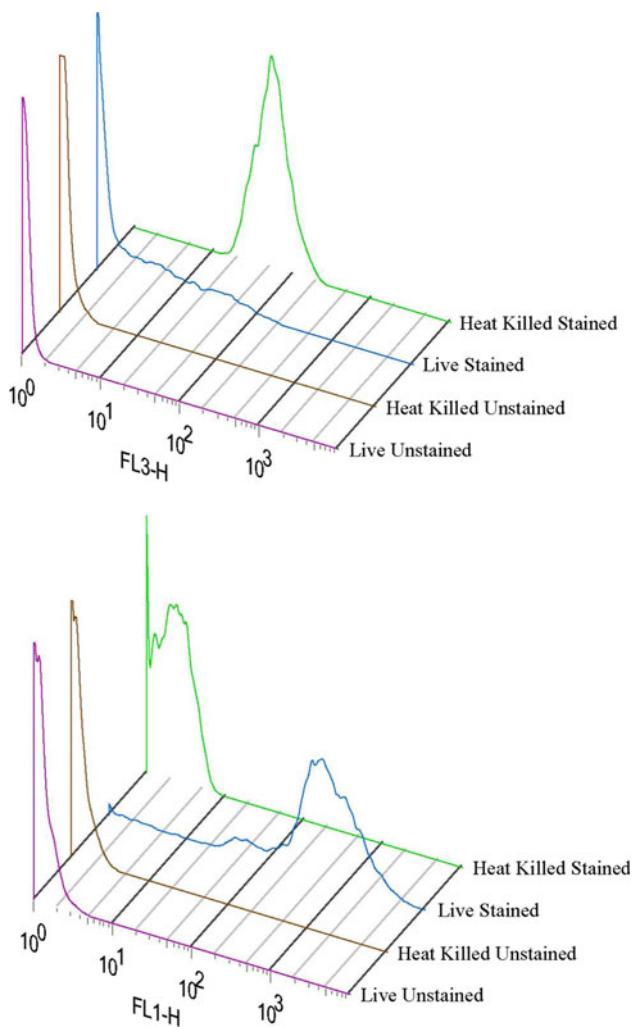


Fig. 4 Histograms comparing the uptake of DiBAC₄(5) (1 µg/ml (1.8 µM), top figure) and CFDA-AM (2.5 µg/ml (4.7 µM), bottom figure) by live and heat-killed *P. anomala* against background signal levels of unstained cells

channel and that from CFDA-AM into the red channel was easily compensated. Caution is required in interpreting the green fluorescence from CFDA-AM because it was frequently found that conditions which ultimately lead to cell death can, at least transiently, promote the development of this fluorochrome. This may be explained by conditions of improved permeability of the cell membrane and agrees with the report that ethanol dramatically improves loading of *Saccharomyces cerevisiae* with BCECF-AM, for example [8].

DiBAC₄(5) was observed to stain heat-killed yeast cells more strongly than the dead population of yeast suffering a more typical demise in culture. Shapiro [18] notes that the oxonols can stain internal cell membranes as well as the plasma membrane, provided they can enter the cell. The intensity of fluorescence, therefore, may not only reflect membrane potential but also the degree of cellular damage.

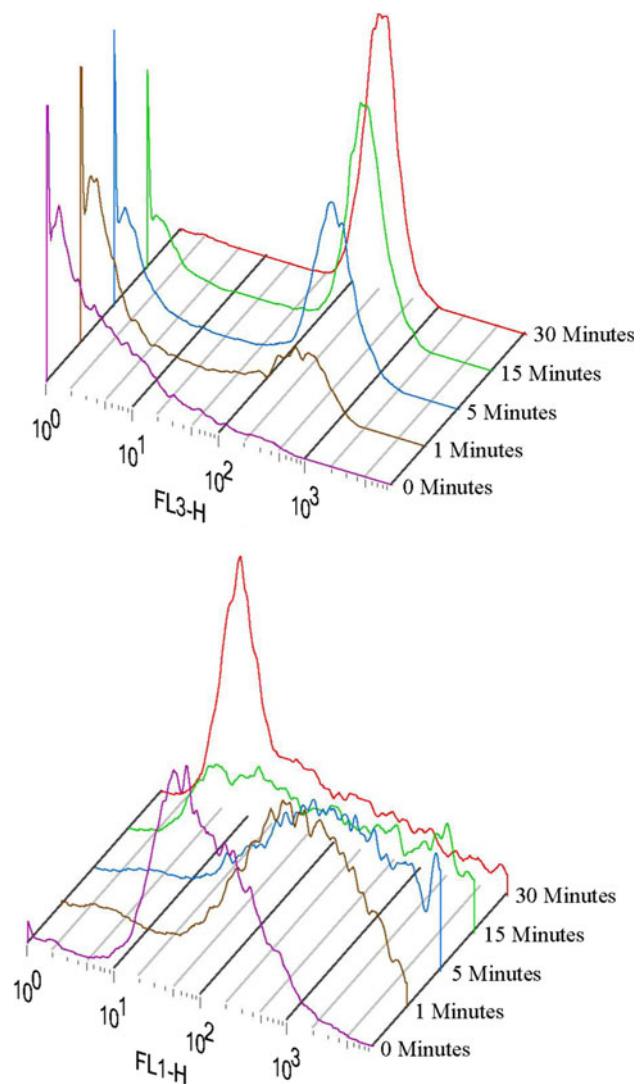
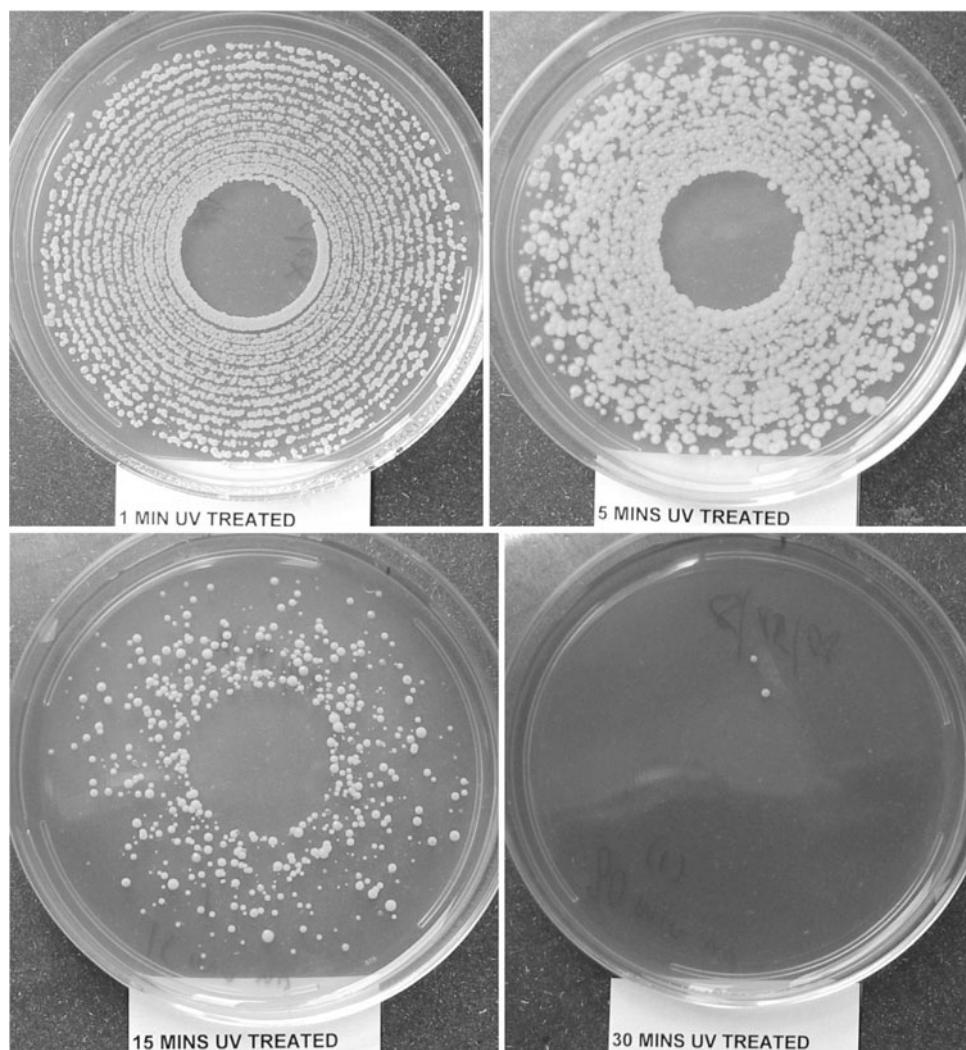


Fig. 5 Histograms of *P. anomala* stained with DiBAC₄(5) (1 µg/ml (1.8 µM), top figure) and CFDA-AM (2.5 µg/ml (4.7 µM), bottom figure) after exposure to UV for 0, 1, 5, 15, and 30 min

DiBAC₄(5) has, perhaps, been less popular than DiBAC₄(3) as a probe for membrane potential because of its higher excitation maximum (590 vs. 493 nm) but the results here show that it can be practically and effectively excited using the 488 nm argon laser line. Furthermore, having longer wavelength emission, it may be more advantageous where high autofluorescence is observed [14].

Oxonol type stains have been employed to monitor yeast viability, and specifically with *P. anomala* to study long-term storage formulations [15]. This study further confirms the potential of flow cytometry to successfully evaluate the physiological status of yeast cultures. Results indicated an increase in permeabilized cells and a decrease in the number of active cells during UV exposure. It also indicated that flow cytometry, used in conjunction with specific

Fig. 6 Effect of UV exposure on culturability of *P. anomala*



probes, could be a useful tool in examining the mechanisms of the action of UV light on yeast viability.

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